



Year: 2019

Genotoxicity assessment of chemical mixtures

More, Simon ; Bampidis, Vasileios ; Benford, Diane ; Boesten, Jos ; Bragard, Claude ; Halldorsson, Thorhallur ; Hernandez-Jerez, Antonio ; Hougaard-Bennekou, Susanne ; Koutsoumanis, Kostas ; Naegeli, Hanspeter ; Nielsen, Søren Saxmose ; Schrenk, Dieter ; Silano, Vittorio ; Turck, Dominique ; Younes, Maged ; Aquilina, Gabriele ; Crebelli, Riccardo ; Gürtler, Rainer ; Hirsch-Ernst, Karen Ildico ; Mosesso, Pasquale ; Nielsen, Elsa ; Solecki, Roland ; Carfi, Maria ; Martino, Carla ; Maurici, Daniela ; Parra Morte, Juan ; Schlatter, Josef

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DOI: <https://doi.org/10.2903/j.efsa.2019.5519>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-180367>

Journal Article

Published Version



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Originally published at:

More, Simon; Bampidis, Vasileios; Benford, Diane; Boesten, Jos; Bragard, Claude; Halldorsson, Thorhallur; Hernandez-Jerez, Antonio; Hougaard-Bennekou, Susanne; Koutsoumanis, Kostas; Naegeli, Hanspeter;

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DOI: <https://doi.org/10.2903/j.efsa.2019.5519>

ADOPTED: 22 November 2018

doi: 10.2903/j.efsa.2019.5519

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Keywords: genotoxicity assessment, chemical mixtures, uncertainty analysis

Requestor: Scientific Committee

Question number: EFSA-Q-2018-00126

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Acknowledgements: The Scientific Committee wishes to thank the following for the support provided to this scientific output: WG member Christiane Vleminckx, Jan van Benthem (hearing expert), Karl Heinz Engel (hearing expert) and Paul White (hearing experts); EFSA staff Paola Manini, Annamaria Rossi, Maria Vittoria Vettori.

Suggested citation: EFSA Scientific Committee, More S, Bampidis V, Benford D, Boesten J, Bragard C, Halldorsson T, Hernandez-Jerez A, Hougaard-Bennekou S, Koutsoumanis K, Naegeli H, Nielsen SS, Schrenk D, Silano V, Turck D, Younes M, Aquilina G, Crebelli R, Gürtler R, Hirsch-Ernst KI, Mosesso P, Nielsen E, Solecki R, Carfi M, Martino C, Maurici D, Parra Morte J and Schlatter J, 2019. Statement on the genotoxicity assessment of chemical mixtures. *EFSA Journal* 2019;17(1):5519, 11 pp. <https://doi.org/10.2903/j.efsa.2019.5519>

ISSN: 1831-4732

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The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.



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1. Introduction

1.1. Background and Terms of Reference as provided by EFSA

Human and ecological risk assessment of combined exposure to multiple chemicals ('chemical mixtures') poses a number of challenges to scientists, risk assessors and risk managers, particularly because of the complexity of the problem formulation, the almost infinite number of possible combinations of chemicals and the large amount of data needed to describe the toxicological profiles and exposure patterns of these chemicals in humans and species present in the environment. The development of harmonised methodologies for combined exposure to multiple chemicals in all areas of EFSA's remit has been identified by EFSA's Scientific Committee as a key priority area. Some EFSA panels and units have initiated activities to support harmonisation of risk assessment methods for both human health and ecology. In particular, the Scientific Committee initiated in 2016 an activity to develop guidance on harmonised risk assessment methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. Work is ongoing and a draft guidance has been published for public consultation in summer 2018 (<https://www.efsa.europa.eu/en/consultations/call/180626-0>). Finalisation of the guidance is expected in spring 2019.

At present, information on the genotoxicity of a chemical drives the type of the assessment in human risk assessment: if the chemical is not genotoxic, a health-based guidance value is usually set, whilst if there is an unavoidable chemical that is a genotoxic carcinogen, the Margin of Exposure approach is usually applied (EFSA, 2005). With respect to assessing the genotoxicity of mixtures, specific additional considerations might be needed, e.g. when it is not possible to fully characterise a complex mixture due to analytical problems.

Different areas within EFSA's remit have different data requirements in relation to the assessment of mixtures:

- Plant protection products:
 - Regulation (EC) No. 1107/2009 on the placing of plant protection products on the market requires that 'interaction between the active substance, safeners, synergists and coformulants shall be taken into account' in the evaluation and authorisation of plant protection products (Article 29).
 - Commission Regulation (EU) No. 283/2013, setting out the data requirements for active substances in plant protection products, in accordance with Regulation (EC) No. 1107/2009 of the European Parliament further requests:
 - a risk assessment of consumer exposure, including, when relevant, a cumulative risk assessment deriving from exposure to more than one active substance;
 - an estimation of the exposure to operators, workers, residents and bystanders including, when relevant, the cumulative exposure to more than one active substance.
 - Commission Regulation (EU) No. 284/2013, setting out the data requirements for plant protection products, in accordance with Regulation (EC) No. 1107/2009 of the European Parliament further requests 'any information on potentially unacceptable effects of the plant protection product on the environment, on plants and plant products shall be included as well as known and expected cumulative and synergistic effects'.
 - Regulation (EC) No. 396/2005 on maximum residue levels (MRLs) of pesticides in or on food and feed of plant and animal origin requires cumulative risk assessment for pesticides to be performed. Recital 6 states: 'It is also important to carry out further work to develop a methodology to take into account cumulative and synergistic effects'. It further specifies that MRLs should be set 'in view of human exposure to combinations of active substances and their cumulative and possible aggregate and synergistic effects on human health'.
- Feed additives:
 - Regulation (EC) No. 429/2008 on the assessment and authorisation of feed additives explicitly addresses risks that may arise from combined exposures if feed additives placed on the market contain more than one (active) ingredient. Annex II lists the requirement that 'when an additive has multiple components, each one may be separately assessed for consumer safety and then consideration given to the cumulative effect (when it can be

shown that there are no interactions between the components). Alternatively, the complete mixture shall be assessed’.

- Smoke flavourings:
 - Regulation (EC) No. 2065/2003 on the assessment and authorisation of smoke flavourings used or intended for use in or on food. Annex II includes the information necessary for the scientific evaluation of primary products, i.e. primary smoke condensates and primary tar fractions produced by controlled thermal degradation of wood in a limited supply of oxygen (pyrolysis), all of these being complex chemical mixtures. In accordance with this Annex, the toxicological data requirements should follow the advice of the Scientific Committee on Food, given in its report of 25 June 1993, according to which relevant data should be generated on the whole mixture.

Legislation in relation to food additives, food contact materials and food contaminants does not have specific provisions requiring risk assessment of mixtures. However, this does not imply that mixtures are never addressed. For example, in Regulation (EC) No. 1881/2006, the setting of maximum levels for certain contaminants in foodstuffs (e.g. dioxins, polycyclic aromatic hydrocarbons and a number of mycotoxins) are underpinned by a mixtures risk assessment.

Given this background, the SC discussed and agreed to develop a statement clarifying how to perform genotoxicity assessment of chemical mixtures with cross-reference to previous EFSA guidance documents.

1.1.1. Terms of Reference

Starting from the basic definition of chemical mixtures as presented in the ‘Guidance on harmonised risk assessment methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals’, the SC should develop a statement that:

- clarifies the peculiarities related to genotoxicity assessment of mixtures, i.e. identification of specific additional considerations and their triggers;
- addresses both component-based and whole mixture approaches.

Consideration from the different areas within EFSA’s remit should be given to explore the feasibility and spectrum of applications of the proposed approaches for human health risk assessment.

1.2. Interpretation of the Terms of Reference and general considerations

A general guidance document addressing harmonised methods for risk assessment of combined exposure to multiple chemicals for all relevant areas within EFSA’s remit, including human health and environmental aspects, has been developed in parallel to this statement and will be published in spring 2019 (2018 draft published for public consultation available at <https://www.efsa.europa.eu/en/consultations/call/180626-0>). Those risk assessment principles are not repeated in this document.

This statement addresses primarily specific issues related to hazard identification of genotoxicity of mixtures and provides a general framework for the assessment of the genotoxic hazard of chemical mixtures. For all other aspects of risk assessment of mixtures, the reader is referred to the guidance document on combined exposure to multiple chemicals in preparation (expected to be published in spring 2019).

It is also not in the scope of this statement to provide guidance on the assays to be applied for genotoxicity testing of chemical mixtures. However the Scientific Committee recommends to follow the opinion on genotoxicity testing strategies (EFSA, 2011) and the statement on ‘Clarification of some aspects related to genotoxicity assessment’ (EFSA, 2017).

Based on the guidance on mixtures, a differentiation is made between mixtures that are chemically fully defined or characterised and mixtures in which not all of the components have been characterised. These two situations are considered separately in this statement.

Examples of chemically fully defined mixtures are a mixture produced by adding together separate chemical substances, a chemically well characterised mixture produced by a controlled process, or a group of separate chemical substances to which combined exposure can occur, such as a group of individual pesticides or food additives. The Scientific Committee notes that the term ‘chemically fully defined’ does not mean that all chemical components have to be known. As with individual chemical

substances, which in practice are never 100% pure, the acceptable impurities in a chemical mixture are usually defined in the specifications.

Considering the wide range of mixtures to be expected, it is not possible to establish a certain generic 'cut-off' value, i.e. the percentage of unidentified chemical substances in a mixture considered acceptable, without further testing (not calling for a whole mixture testing or testing of the respective fraction). The Scientific Committee, however, stresses that state-of-the-art analytical methodologies should be applied. Taking into account the nature of the mixture (e.g. chemical classes of the constituents or its production process), the employed analytical techniques should be able to detect and to quantify constituents at limits of detection (LOD) and limits of quantification (LOQ), respectively, generally accepted in routine analysis. So, an accepted analytical 'cut-off' value for a mixture of volatiles directly amenable to gas chromatography (GS) analysis may differ substantially from that for a mixture of non-volatiles from different chemical classes analysed via liquid chromatography/mass spectrometry (LC/MS). Therefore, in the different areas within EFSA's remit, specific considerations are needed (e.g. for botanicals, novel foods, pesticides or food and feed additives).

The Scientific Committee notes that it may be possible to deviate from approaches proposed in the present statement, if it can be scientifically justified.

Definitions of the terms used in this statement are given in the glossary in the end of this document.

2. Assessment

2.1. Chemical characterisation of mixtures

The demonstration of the identity and stability (batch-to-batch variability as well as stability over time) of a mixture is always required to ensure that the mixture tested is representative of the mixture to be placed on the market (e.g. for regulated products) or representative for mixtures present in the environment or food (e.g. contaminants).

2.1.1. Qualitative and quantitative analysis of the composition of a mixture

The first step must be to characterise the mixture as fully as possible. Compositional data are required for qualitative and quantitative analysis of a mixture. Although the characterisation of mixtures is relevant also for other toxicity aspects, it is particularly important for the assessment of genotoxicity.

Chemically fully defined mixtures

For mixtures of chemically defined substances, information on the identities and the relative ratios should be provided. For mixtures prepared by adding individual chemical substances, the decision on which degree of 'purity' of the individual components can be considered sufficient does not differ from the decision to be taken for individual chemical substances. This may also depend on specific sources and the production process of the mixture.

Mixtures containing a substantial fraction of unidentified components

For mixtures for which not all components have been chemically fully identified, a quantitative characterisation of the main constituents should be performed, at least for sum parameters (e.g. total phenols, total acids, total protein or reducing sugars). The percentage of unidentified components should be indicated and should be as low as possible. Therefore, the analytical methods employed to characterise the mixture should at least be able to cover the type and the expected analytes (i.e. chemical substances that, based on knowledge of the source and the production/formation of the mixtures, are expected to be potentially present).

2.2. Genotoxicity assessment of chemically fully defined mixtures

For chemically fully defined mixtures, the Scientific Committee recommends applying a component-based approach, i.e. assessing all components individually using all available information including read across and quantitative structure–activity relationship (QSAR) considerations about their genotoxic potential, following the Scientific Committee guidance (EFSA, 2011, 2017). This means that for regulated products, conclusions on genotoxicity will be required for all components or at least for representative chemical substances for mixtures containing structurally related substances. For chemically fully defined mixtures made of closely related molecules, for which no divergent genotoxic

potentials are anticipated based on structure–activity relationship (SAR) considerations, the testing of the whole mixtures may also be acceptable, as no dilution of the effect is expected when all components in a mixture share the same properties. However, when dealing with mixtures of structural isomers, the genotoxic potential might differ between the structural isomers and this should be addressed case by case.

If such a mixture contains one or more chemical substances that are assessed to be genotoxic *in vivo* via a relevant route of administration (i.e. in most cases after oral exposure), the whole mixture raises concern for genotoxicity. The risk to human health related to this identified hazard may need to be taken into account in the overall risk assessment.

For mixtures that contain individual components indicating a potential concern for genotoxicity but for which the data available are not sufficient to conclude on genotoxicity, e.g. positive results in *in vitro* genotoxicity tests of an individual component without an appropriate *in vivo* follow-up test, additional data would be needed to complete an assessment, following the Scientific Committee guidance (EFSA, 2011, 2017).

When the mixture contains structurally related chemical substances, a representative chemical substance (ideally expected to have the highest DNA reactivity among the structurally related substances, based on expert judgement), could be further tested and used as an indicator substance for all structurally related chemical substances. This should be carried out as for individual chemical substances following the Scientific Committee guidance (EFSA, 2011, 2017). If a fraction or a representative chemical substance of the mixture is tested, the reasons for the choice need to be explained.

The Scientific Committee reiterates its earlier statement that chemical substances that are both genotoxic and carcinogenic should not be deliberately added to foods or used earlier in the food chain. In certain cases, i.e. unavoidable contaminants and impurities, it might be possible to conclude that human exposure is likely to be of low concern from a public health perspective. Such a conclusion may be reached based on a Margin of Exposure (MOE) approach (EFSA, 2005, 2012a) when respective carcinogenicity data are available, either for the genotoxicant itself or for a structurally closely related chemical substance. For details on the application of the MOE approach for mixtures, the reader is referred to the guidance document on combined exposure to multiple chemicals under development (EFSA in preparation, expected to be published in spring 2019). The Scientific Committee notes that in the scientific community there is, as yet, no consensus on whether and how a MOE approach could be applied to genotoxicity data alone (in the absence of relevant carcinogenicity data).

If no relevant carcinogenicity data are available and the estimated exposure to the chemical substance is very low, it might be possible to apply the Threshold of Toxicological Concern (TTC) concept (EFSA, 2012b; and EFSA ongoing revision of TTC guidance, expected to be published in spring 2019).

2.3. Genotoxicity assessment of mixtures containing a substantial fraction of unidentified components

If a mixture contains, besides chemically identified substances, a substantial¹ fraction of chemical substances that have not been chemically characterised, the Scientific Committee recommends that first the chemically defined substances be assessed individually for their potential genotoxicity, using all available information, including read across and QSAR considerations about their genotoxic potential, following the Scientific Committee guidance (EFSA, 2011, 2017). This means that for regulated products, a conclusion on genotoxicity will be required for all identified components or at least for representative chemical substances for mixtures containing structurally related substances. As described in Section 2.2, if the mixture contains one or more chemical substances that are evaluated to be genotoxic *in vivo* via a relevant route of administration, the whole mixture raises concern about genotoxicity.

As already mentioned in Section 2.2, for mixtures that contain individual components that may indicate a potential concern for genotoxicity but for which the data available are not sufficient to conclude on genotoxicity, e.g. positive results in *in vitro* genotoxicity tests of an individual component, additional data would be needed to complete an assessment (EFSA, 2011, 2017).

If none of the identified chemical substances in a mixture raises concern for genotoxicity, the genotoxic potential of the unidentified fraction should also be evaluated to complete the assessment of

¹ A general definition of 'substantial' is not possible as it depends on several factors such as the nature of the source and the production/formation process and needs to be decided on a case-by-case basis.

the mixture. Experimental testing of the unidentified fraction should be considered as the first option or, if this is not feasible and a scientific justification is provided, testing of the whole mixture should be undertaken. Further fractionation of the test material could be considered case by case to remove inert, toxicologically irrelevant components from the mixture (e.g. high-molecular-weight polymers) to minimise the dilution of the components of interest in the tested sample, or to remove highly toxic components (e.g. surface active substances) that may prevent testing adequately high doses of the mixture because of overt toxicity. Moreover, if either the starting material used or the production process indicates the possible presence of genotoxins in the unidentified fraction of the mixture, an attempt should be made to isolate and test the fraction of concern as such, if it is not possible to chemically identify and quantify the substance.

The testing strategy for a whole mixture or its fraction(s) should follow the Scientific Committee testing strategy guidance for individual chemical substances (EFSA, 2011, 2017). However, as mentioned in the OECD (2015, 2016a, 2016c; Test Nos. 473, 476, 487, 490) (*in vitro* testing): 'When the test chemical is not of defined composition, e.g. substance of unknown or variable composition, complex reaction products or biological materials (i.e. UVCBs), environmental extracts, etc., the top concentration may need to be higher (e.g. 5 mg/mL), in the absence of sufficient cytotoxicity, to increase the concentration of each of the components'.

If testing of the whole mixture or its fraction(s) in an adequately performed set of *in vitro* assays (e.g. (OECD, 1997) Test No. 471 and (OECD, 2016e) Test No. 487) following the Scientific Committee testing strategy (EFSA, 2011, 2017) provides clearly negative results, the mixture could be considered as of no concern with respect to genotoxicity and no further testing is recommended.

If testing of the whole mixture or its fraction(s) in an adequately performed set of *in vitro* assays provides one or more positive results, *in vivo* follow-up testing should be considered to assess the relevance of these findings for risk assessment. The follow-up study should be tailored case by case based on the activity profile/mode of action observed *in vitro*, following the Scientific Committee genotoxicity testing strategy (EFSA, 2011, 2017), and taking into account any other relevant information (e.g. on source and chemical characteristics of the mixture).

If the *in vivo* testing of an *in vitro* positive mixture provides negative results, the relevance of the findings obtained in the *in vivo* follow-up tests will depend on the genetic effect assessed (i.e. gene mutations, structural or numerical chromosomal aberrations), the test protocol applied (route of exposure, tissues, etc.) and expert judgement on the reliability of the results obtained (including consideration of target tissue exposure).

In some instances it can be anticipated that negative results in the follow-up tests can support, with sufficient confidence, a lack of concern about the *in vivo* genotoxicity of the mixture. For example, for a mixture that is directly clastogenic *in vitro*, a robust assessment *in vivo* could be performed by applying a mammalian alkaline comet assay (OECD (2016d) Test No. 489) to several tissues, including the site of first contact, to animals in which the mixture was administered orally. For other effects, such as induction of gene mutations and/or clastogenicity *in vitro* following metabolic activation, the assessment of systemic genotoxic effects (e.g. in the liver or bone marrow) may be limited by the fact that target tissue exposure cannot be demonstrated, as any toxic effect elicited in the target tissue by the mixture cannot be unequivocally attributed to the (*in vitro*) genotoxic component. In this scenario, the conclusion drawn would have a higher uncertainty.

Another relevant concern is the follow-up testing of *in vitro* aneugens. At present, the only validated methodology to assess aneugenicity is the rodent bone marrow micronucleus assay (OECD (2016b) Test No. 474). In this scenario, the lack of information on target tissue exposure may be a critical limitation, also because of the possibility of effects at the site of first contact, in which local concentrations may be higher than in the bone marrow and aneugenic effects cannot be investigated reliably because micronucleus assays in tissues other than bone marrow or peripheral blood are not sufficiently validated as yet.

So, for negative results in the *in vivo* follow-up study, the possible limitations of *in vivo* testing should be weighed in an uncertainty analysis before reaching a conclusion of no concern with respect to genotoxicity of complex mixtures that provided positive *in vitro* results.

Conversely, for positive results in the *in vivo* follow-up tests, it can be concluded that the mixture does raise a concern about genotoxicity. In this scenario, it may also be prudent to consider in the overall assessment positive test results *in vivo* that are obtained under conditions associated with overt toxicity, which are usually considered of limited relevance, as it cannot be decided whether the observed genotoxic effects are secondary due to cytotoxicity. The underlying cytotoxicity (organ toxicity) could be elicited by the same or different components of the mixture.

3. Conclusions

In this statement, a differentiation is made between mixtures that are chemically fully defined or characterised and mixtures in which not all the components have been characterised. The Scientific Committee however notes that the term 'chemically fully defined' does not mean that all chemical components have to be known. As with individual chemical substances, which in practice are never 100% pure, the acceptable impurities in a chemical mixture are usually defined in the specifications based on expert judgement:

- The first step must be to characterise the mixture as fully as possible. Compositional data are required for qualitative and quantitative analysis of a mixture.
- The demonstration of the identity and stability of a mixture is always required to ensure that the mixture tested is representative of the mixture to be placed on the market or representative for mixtures present in the environment or food.
- For chemically fully defined mixtures, the Scientific Committee recommends applying a component-based approach, i.e. assessing all components individually using all available information including read across and QSAR considerations about their genotoxic potential.

If a mixture contains one or more chemical substances that are assessed to be genotoxic *in vivo* via a relevant route of administration, the mixture raises concern with respect to genotoxicity.

If the assessment of all components of a chemically fully defined mixture results in the conclusion that none of these raises a concern with respect to genotoxicity, the mixture is also considered of no concern with respect to genotoxicity.

For mixtures that contain individual components indicating a potential concern for genotoxicity but for which the data available are not sufficient to conclude on genotoxicity, additional data would be needed to complete an assessment.

If a mixture contains, besides chemically identified substances, a substantial fraction of chemical substances that have not been chemically identified, the Scientific Committee recommends that, first, the chemically identified substances be assessed individually for their potential genotoxicity, using all available information, including read across and QSAR considerations about their genotoxic potential.

If none of the identified chemical substances in a mixture raises concern for genotoxicity, the genotoxic potential of the unidentified fraction should also be evaluated to complete the assessment of the mixture. Experimental testing of the unidentified fraction should be considered as the first option or, if this is not feasible and a scientific justification is provided, testing of the whole mixture should be undertaken, following the Scientific Committee guidance for individual chemical substances (EFSA, 2011, 2017):

- If testing of the whole mixture or fractions containing the unidentified substances in an adequately performed set of *in vitro* assays provides clearly negative results, the mixture should be considered as of no concern with respect to genotoxicity and no further testing is recommended.
- If testing of the whole mixture or fractions containing the unidentified substances in an adequately performed battery of *in vitro* assays provides one or more positive results, an *in vivo* follow-up study should be considered to assess the relevance of the findings for risk assessment.
- For negative results in the *in vivo* follow-up tests of positive results in *in vitro* assays, the relevance of the findings obtained in the *in vivo* follow-up tests will depend on the genetic effect assessed (i.e. gene mutations, structural or numerical chromosomal aberrations or any other effect), the test protocol applied (route of exposure, tissues, etc.) and expert judgement on the reliability of the results obtained (including consideration of target tissue exposure).
- For positive results in the *in vivo* follow-up tests of positive results in *in vitro* assays, the mixture does raise a concern about genotoxicity.

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Glossary and Abbreviations

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| Component-based approach | An approach in which the risk of a group of chemical substances is assessed based on exposure and effect data of its individual components |
| GS | Gas Chromatography |
| LC/MS | liquid chromatography/mass spectrometry |
| limit of detection (LOD) | Lowest concentration of a chemical substance in a defined matrix in which positive identification can be achieved using a specified method |
| limit of quantification (LOQ) | Lowest concentration of a chemical substance in a defined matrix in which positive identification and quantitative measurement can be achieved using a specified analytical method |
| Margin of Exposure (MOE) | Ratio of (a) a reference point of (eco)toxicity to (b) the theoretical, predicted or estimated exposure dose or concentration |
| Mixture | Any combination of two or more chemical substances, regardless of source and spatial or temporal proximity that may contribute to effects |
| Mode of Action | Biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. It refers to the major steps leading to an adverse health effect following interaction of the chemical substance with biological targets. It does not imply full understanding of mechanism of action at the molecular level |
| MRL | maximum residue level |

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| QSAR | quantitative structure–activity relationship |
| SAR | structure–activity relationship |
| Substance | A chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used, but excluding any solvent that may be separated without affecting the stability of the substance or changing its composition |
| Sum parameters | Parameters determining the content of classes of chemical substances with common structural aspects (e.g. phenols, proteins or reducing sugars) rather than individual constituents |
| TTC | Threshold of Toxicological Concern |
| Uncertainty | A general term referring to all types of limitations in available knowledge that affect the range and probability of possible answers to an assessment question. Available knowledge refers here to the knowledge (evidence, data, etc.) available to assessors at the time the assessment is conducted and within the time and resources agreed for the assessment. Sometimes uncertainty is used to refer to a source of uncertainty and sometimes to its impact on the conclusion of an assessment (EFSA, 2018). |
| UVCBs | Substances of Unknown or Variable composition, Complex reaction products or Biological materials |
| Whole mixture approach | A risk assessment approach in which the mixture is treated as a single entity, similar to single chemical substances and so requires dose–response information for the whole mixture of concern |